Antimicrobial Agents and Chemotherapy, June 2011, p. 2623–2628 Vol. 55, No. 6
0066-4804/11/$12.00 doi:10.1128/AAC.01374-10

Comparative Efficacies of Candidate Antibiotics against *Yersinia pestis* in an *In Vitro* Pharmacodynamic Model[▽]

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Received 6 October 2010/Returned for modification 2 March 2011/Accepted 30 March 2011

***Yersinia pestis*, the bacterium that causes plague, is a potential agent of bioterrorism. Streptomycin is the "gold standard" for the treatment of plague infections in humans, but the drug is not available in many countries, and resistance to this antibiotic occurs naturally and has been generated in the laboratory. Other antibiotics have been shown to be active against *Y. pestis in vitro* and *in vivo*. However, the relative efficacies of clinically prescribed regimens of these antibiotics with streptomycin and with each other for the killing of *Yersinia pestis* are unknown. The efficacies of simulated pharmacokinetic profiles for human 10-day clinical regimens of ampicillin, meropenem, moxifloxacin, ciprofloxacin, and gentamicin were compared with the gold standard, streptomycin, for killing of *Yersinia pestis* in an *in vitro* pharmacodynamic model. Resistance amplification with therapy was also assessed. Streptomycin killed the microbe in one trial but failed due to resistance amplification in the second trial. In two trials, the other antibiotics consistently reduced the bacterial densities within the pharmacodynamic systems from 10^8 CFU/ml to undetectable levels ($<10^2$ CFU/ml) between 1 and 3 days of treatment. None of the comparator agents selected for resistance. The comparator antibiotics were superior to streptomycin against *Y. pestis* and deserve further evaluation.**

Yersinia pestis is the causative agent of plague. Rodents are the natural reservoir for *Y. pestis*, but this microbe can be transmitted to humans through the bite of an infected flea, resulting in bubonic, septicemic, and pneumonic plagues (1, 3, 25). Untreated bubonic plague is associated with a 40% mortality rate, while untreated septicemic and pneumonic plagues are both associated with 100% mortality rates (9). Streptomycin is considered the "gold standard" for the treatment of all forms of plague. A regimen of streptomycin (1 g given intramuscularly or intravenous every 12 h for 10 days), usually in combination with other antimicrobial agents, reduces the mortalities of bubonic, septicemic, and pneumonic plagues to 14, 22, and 57%, respectively (6).

Y. pestis has been used as a bioweapon and has the potential to be used as an agent of bioterrorism (17). Furthermore, *Y. pestis* isolates that are resistant to streptomycin have been generated in the laboratory and exist in nature (13, 20, 27). Thus, there is a need to identify other drugs that have activity against *Y. pestis*.

If it were used as an agent of bioterrorism, it is likely that the microbe would be disseminated via aerosol, resulting in inhalation-induced pneumonic plague (17). Gentamicin, ciprofloxacin, moxifloxacin, ampicillin, meropenem, and doxycycline demonstrate *in vitro* activity against *Y. pestis* and have demonstrated efficacy in murine infection models (2, 5, 12, 28). Gentamicin, doxycycline, and ciprofloxacin have been successful in the treatment of human plague (3, 18, 23).

Since naturally occurring pneumonic plague is rare and it is unethical to intentionally infect people with this potentially deadly bacterium, controlled, randomized, double-blinded comparative clinical trials to delineate the relative efficacies of these antibiotics can never be conducted. Thus, we used an *in vitro* hollow-fiber pharmacodynamic model (HFPM) to define the efficacies of simulated clinical regimens for gentamicin, ciprofloxacin, moxifloxacin, ampicillin, meropenem, and doxycycline relative to that of the "gold standard" streptomycin, for the killing of a pan-susceptible *Y. pestis* isolate and for the prevention of emergence of resistance during therapy.

MATERIALS AND METHODS

Bacterium. *Y. pestis* strain ΔCO92, an avirulent strain that maintains the same growth rate as the virulent progenitor (unpublished data), was used. The microbe was stored in 10% glycerol at −80°C. For each study, a sample of the bacterial stock was streaked onto blood agar and was incubated for 48 h at 35°C. Colonies were passed onto fresh blood agar and were grown overnight. Bacterial suspensions were made by directly suspending colonies from the overnight culture into medium. The bacterial suspensions were diluted to the desired concentrations with medium and were used immediately. The bacterial concentrations of the suspensions were confirmed by quantitative cultures.

Susceptibility and mutation frequency studies. Macrodilution broth and agar dilution MICs and minimal bactericidal concentrations (MBCs) were determined for streptomycin, gentamicin, ciprofloxacin, moxifloxacin, ampicillin, meropenem, and doxycycline (8). Mutation frequencies (to 2× and 3× MIC) for each antibiotic were also determined.

Comparative antibiotic efficacy studies in an *in vitro* HFPM. The HFPM has been described previously (9, 19–20). *Y. pestis* were inoculated into the extracapillary space of HFPM experimental arms at 10^8 CFU/ml (15 ml) to replicate the total bacterial inoculum that was used by McCrumb et al. (22) in their nonhuman primate models of bubonic/septicemic and pneumonic plague. This bacterial burden also approximated the total number of bacteria that has been documented in the bloodstream of humans with severe plague infections (4). The bacteria in the HFPM experimental arms were exposed to the fluctuating con-

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▽ Published ahead of print on 12 April 2011.

TABLE 1. Targeted pharmacokinetic-pharmacodynamic parameters for simulation in hollow-fiber experimental arms[a]

Treatment arm	AUC/MIC	C_{max}/MIC	Trough/MIC	Time > MIC (h)	% time above MIC
Streptomycin, 1 g q12h	151.51	19.09	0.90	23	96
Gentamicin, 5 mg/kg q24h	170.28	15.67	2.25	24	100
Ciprofloxacin, 500 mg q12h	898.05	80.24	13.06	24	100
Moxifloxacin, 400 mg q24h	508.14	38.38	10.17	24	100
Doxycycline, 100 mg q12h	38.03	2.01	1.31	24	100
Meropenem, 1 g q8h	12141.98	2023.33	15.81	24	100
Ampicillin, 2 g q6h	2291.85	291.36	9.10	24	100

[a] The MICs for the antibiotics examined are shown in Table 2.

centrations of the antibiotics that simulated the mean serum pharmacokinetic profiles of the drugs that were reported in humans. The simulated mean serum concentration-time profiles were for the clinical regimens of gentamicin (5 mg/kg of body weight intravenously [i.v.] every 24 h (Q24h), streptomycin (1 g i.v. Q12h), ciprofloxacin (500 mg orally [p.o.] Q12h), moxifloxacin (400 mg p.o. q24h), ampicillin (2 g i.v. Q6h), meropenem (1 g i.v. q8h), and doxycycline (100 mg p.o. q12h) (following a 200-mg doxycycline loading dose) (Table 1). The targeted pharmacokinetic parameters were for the free (non-protein-bound) fraction of these regimens (24). Another HFPM arm served as a no-treatment control. Antibiotics were given for 10 days. Throughout an experiment, bacterial samples were removed from each HFPM arm and were replaced with the same volume of fresh medium. Washed bacterial samples were quantitatively cultured on antibiotic-free agar and agar supplemented with 2× to 3× MIC of the corresponding treatment drug to assess the effect of that antibiotic on the killing of the total bacterial population and for resistance amplification. The lower limit of quantification for the culture assay was 50 CFU/ml. Serial medium samples were collected from the HFPM arms for measurement of drug content by liquid chromatography/tandem mass spectrometry (LC/MS/MS) to validate that the targeted pharmacokinetic profiles were simulated. At the end of the 10-day study, the total volume of bacterial suspensions in the HFPM arms that were culture negative on day 8 were quantitatively cultured on drug-free agar to determine whether the culture-negative arms were sterile. The increases in antibiotic MICs between the colonies of bacteria that grew on antibiotic-supplemented agar and the parent isolate were determined. The comparative HFPM studies were conducted twice.

Time-kill study of *Y. pestis* and doxycycline. Forty-eight-hour time-kill studies were conducted with *Y. pestis* to assess the effect of 0.25× to 2× MIC concentrations of doxycycline on the killing of this microbe. The starting inoculum of approximately 10^8 CFU/ml (15 ml) of bacterium replicated the starting concentration of *Y. pestis* used in the HFPM. Bacteria and different doxycycline concentrations were added to flasks, which were incubated at 35°C in a water-shaker bath. Medium and drug were changed every 24 h. Quantitative cultures were conducted on washed bacterial samples at the 0-, 24-, and 48-h time points.

RESULTS

Susceptibility and mutation frequency studies. The MICs, MBCs, and mutation frequencies of 2× to 3× the baseline MICs of the antibiotics are shown in Table 2. The MIC values for the parent *Y. pestis* isolate were the same as the MBCs for ciprofloxacin, moxifloxacin, ampicillin, and meropenem. For gentamicin and streptomycin, the MBCs were 1 dilution higher than the respective MICs. Doxycycline was bacteriostatic since the MBC of >64 mg/liter was at least >32× the MIC value of 2 mg/liter. The mutation frequencies were slightly higher at 2× MIC than at 3× MIC for each antibiotic.

TABLE 2. Macrodilution broth MIC and MBC results for *Y. pestis* ΔCO92 and mutation frequencies at 2× and 3× MIC of the respective antibiotics[a]

Drug	MIC (mg/liter)	MBC (mg/liter)	Mutation frequencies (log CFU)	
			2× MIC	3× MIC
Streptomycin	2	4	−6.55 to −7.78	−7.95 to −8.95
Gentamicin	0.5	1	−6.03 to −7.85	−6.94 to −8.95
Ciprofloxacin	0.03	0.03	−7.90 to −8.81	−8.30 to −9.12
Moxifloxacin	0.06	0.06	−6.21 to −8.81	−8.12 to −9.20
Doxycycline	2	>64	−5.09 to −6.10	−5.38 to −5.97
Meropenem	0.06	0.06	−7.54 to −8.18	−8.30 to −8.82
Ampicillin	0.25	0.25	<−8.38	−8.81 to −9.42

[a] The susceptibility studies were conducted in duplicate on four separate days.

HFPM comparative antibiotic efficacy studies. In the first trial, the HFPM arm treated with the simulated concentration-time profile for streptomycin (1 g i.v. q12h) showed an initial decrease in bacterial density from 10^8 to approximately 5×10^4 CFU/ml on day 1, followed by regrowth such that the bacterial densities matched those of the control arm by day 3 (Fig. 1A). In the second trial, streptomycin sterilized the HFPM arm (Fig. 1B). Subpopulations of *Y. pestis* in the control arm increased in proportion to the total population, showing that microbes with decreased susceptibilities to the treatment antibiotics were present in the bacterial inoculum at the start of therapy (Fig. 2A).

Regrowth (and treatment failure) of streptomycin therapy in the first trial was due to rapid amplification of the drug-resistant subpopulation (Fig. 2B) that was present in the bacterial suspension prior to initiation of antibiotic therapy (Table 3). These mutants had MICs to streptomycin of >256 mg/liter, compared to an MIC of 2 mg/liter for the parent strain. The streptomycin-resistant mutants did not show cross-resistance to gentamicin, since the isolates with very high MICs to streptomycin and the parent *Y. pestis* strain all had MICs to gentamicin of 0.5 mg/liter. In contrast, in trial 2 (in which streptomycin therapy was successful), colonies that grew on agar that was supplemented with this antibiotic had MICs to streptomycin that were 4 to 8 mg/liter. These isolates did demonstrate decreased susceptibilities to gentamicin (gentamicin MICs of 1 to 2 mg/liter) (Table 3).

In two trials, gentamicin, ampicillin, meropenem, ciprofloxacin, and moxifloxacin consistently and rapidly killed the drug-susceptible *Y. pestis* population and did not amplify the subpopulations with decreased susceptibilities to these antibiotics (Fig. 1 and 2C through 2H). Doxycycline was ineffective in our HFPM at the dose examined, despite its proven effectiveness in *in vivo* experimental and clinical studies (5, 23). Approximately 10^2 CFU/ml of mutants resistant to 2× MIC of doxycycline were present in the HFPM systems at initiation of therapy (Fig. 2H). These mutants were amplified with doxycycline therapy. Time-kill studies showed that the lack of response of *Y. pestis* was not due to the HFPM systems, since doxycycline exposures higher than those used in the HFPM had no effect or a marginal effect against *Y. pestis* in the time-kill studies (Fig. 3).

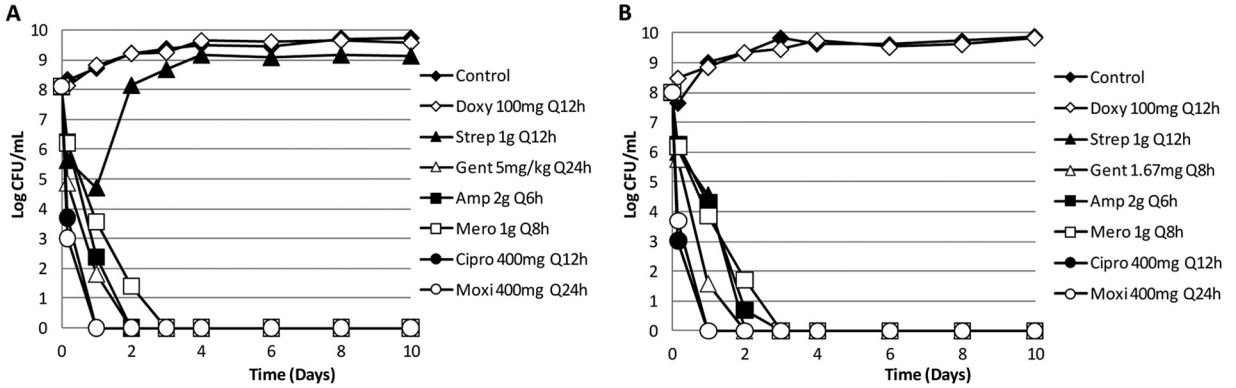

FIG. 1. Antimicrobial effect of simulated clinical regimens for streptomycin, gentamicin, ciprofloxacin, moxifloxacin, ampicillin, meropenem, and doxycycline against *Y. pestis* in an *in vitro* hollow fiber pharmacodynamic model (HFPM). Effects of these antibiotic regimens on the total bacterial population in two HFPM trials (trial 1 [A] and trial 2 [B]).

DISCUSSION

Because naturally occurring human disease due to *Y. pestis* is uncommon and it is unethical to intentionally infect people with a bacterium that can cause severe morbidity and mortality, it is impossible to conduct controlled, randomized, double-blinded comparative clinical trials to define the relative efficacies of different antibiotics for the treatment of human plague. Furthermore, the half-lives of drugs in small animals are frequently shorter than those in humans. Thus, animal models may underestimate the true antimicrobial efficacy of some drugs, and this makes it difficult to determine the relative efficacies of different antibiotic classes (11). The serum concentration-time profiles of antibiotics can be accurately simulated within HFPMs. This enabled our group to conduct the only study that has compared the relative antimicrobial efficacies of clinically prescribed regimens with the

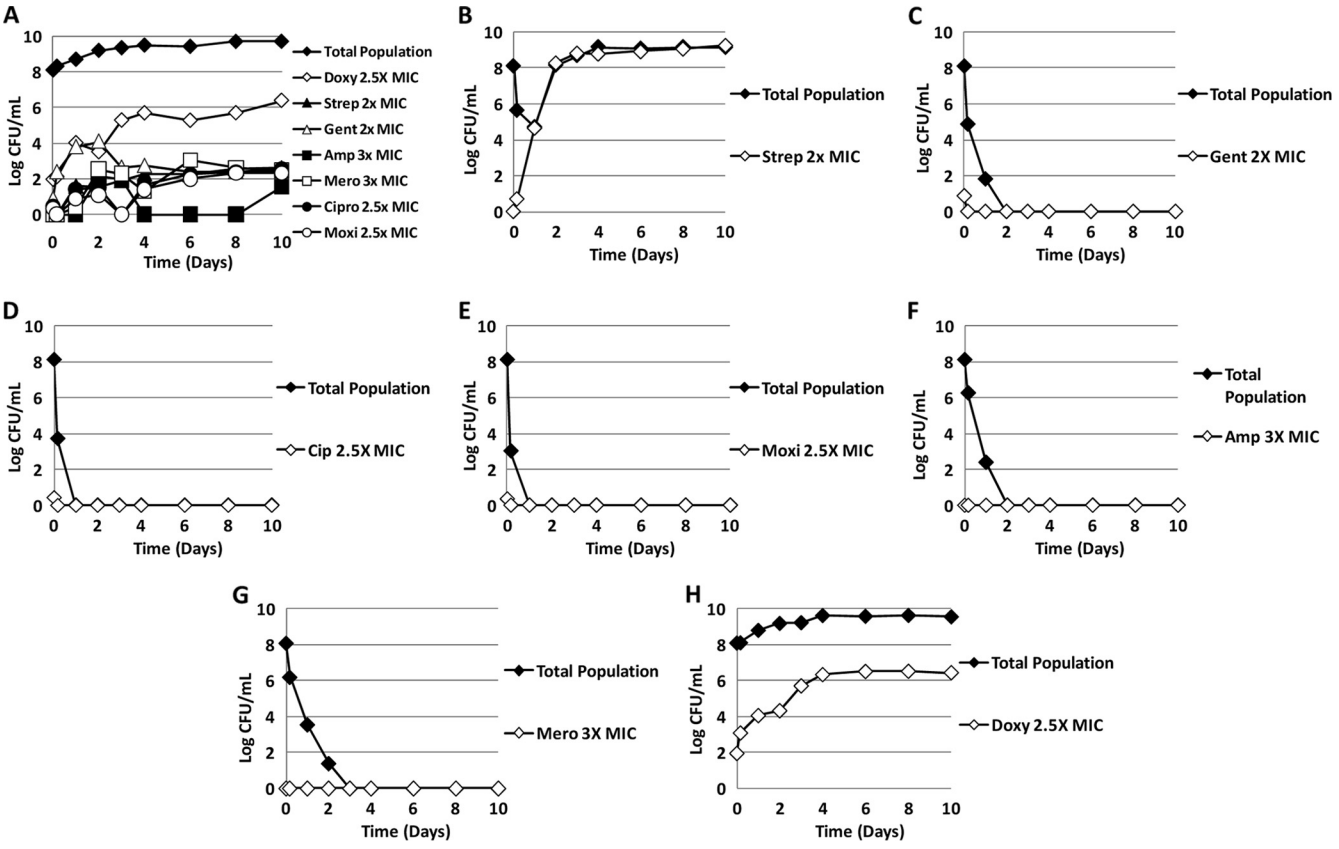

FIG. 2. Effects of individual antibiotic regimens in the HFPM on the total population and the population with decreased susceptibility to the administered drugs in trial 1 (effects of drug regimens on the total bacterial populations are shown in Fig. 1A).

TABLE 3. Streptomycin MICs for *Y. pestis* isolates that grew on streptomycin-supplemented agar plates from nontreatment control and streptomycin treatment arms in two hollow-fiber trials[a]

Trial	Bacterial group	Streptomycin MIC (mg/liter) on day:		
		0	6	10
1	Nontreatment control	>256[b]	>256[b]	>256[b]
	Streptomycin treatment arm	>256[b]	>256[b]	>256[b]
2	Nontreatment control	4–8[c]	4–8[c]	4–8[c]
	Streptomycin treatment arm	4–8	NG[d]	NG

[a] Streptomycin therapy failed in trial 1 but was successful in trial 2. The wild-type *Y. pestis* isolate had a streptomycin MIC of 2 mg/liter, and the antibiotic-supplemented agar contained 4 mg/liter of streptomycin.

[b] These isolates had gentamicin MICs of 0.5 mg/liter, identical to the gentamicin MIC for the wild-type *Y. pestis* strain.

[c] These isolates had gentamicin MICs of 1 to 2 mg/liter compared to a gentamicin MIC for the wild-type strain of 0.5 mg/liter.

[d] NG, no growth on agar supplemented with streptomycin at 2× the MIC of the wild-type strain.

"gold standard," streptomycin, and other commercially available antibiotics on the killing of a pan-antibiotic-susceptible isolate and on resistance amplification.

In our *in vitro* HFPM studies, the efficacies of ampicillin, meropenem, ciprofloxacin, moxifloxacin, and gentamicin were superior to that of the "gold standard," streptomycin. These antibiotics consistently and rapidly sterilized the HFPM arms, while streptomycin failed in one of two trials due to amplification of resistance. The failure of streptomycin in the *in vitro* HFPM was concordant with the results reported by McCrumb et al. (22), who reported that 2 of 7 nonhuman primates with experimental plague pneumonia failed streptomycin therapy due to emergence of resistance. Emergence of resistance has not been reported in murine models of plague pneumonia (2), perhaps because the *Y. pestis* inoculum of 2.6 to 4.6 log CFU/mouse used in murine models of septicemic and inhalation-induced plague pneumonia were less than the streptomycin mutation frequency of resistance of -6.55 to -7.78 log CFU for this microbe. Furthermore, the inoculum examined in mice was substantially lower than the 1.5×10^9 CFU of bacteria that were examined in our HFPM arms. The inoculum used in the HFPM experiments approximated the 2×10^{11} CFU total burden of *Y. pestis* that has been documented in the bloodstream of some people with severe plague (based on a report that up to 4×10^7 CFU/ml of *Y. pestis* can be cultured from the blood of humans with plague septicemia [4] and the estimate that the mean blood volume in a human male is approximately 5 liters [15]). Also, the starting inoculum used in the HFPM studies approximated the bacterial challenge used by McCrumb and colleagues in their nonhuman primate models of severe bubonic-septicemic and pneumonic plagues (22).

Doxycycline, the only other drug (besides streptomycin) that is approved by the U.S. FDA for the treatment of plague infections, could not be assessed in the *in vitro* HFPM since *Y. pestis* exposed to a simulated clinical regimen for this drug showed the same growth profile as the no-treatment controls. This finding is consistent with our previous findings in immune-normal and neutropenic murine models of plague pneumonia using the fully virulent *Y. pestis* strain CO92. That study demonstrated that this bacteriostatic drug required the presence of neutrophils in order to optimize treatment benefit. In contrast, the bactericidal drug gentamicin provided the same microbiological effect in immune-normal and neutropenic mice (16).

The gentamicin and streptomycin regimens simulated in our studies had similar free area under the concentration-time curve (AUC)/MIC, maximum concentration of drug in serum (C_{max})/MIC, and time-above-MIC ($T>$MIC) values (Table 1) and similar mutation frequency values (Table 2). Yet gentamicin was successful in two trials, while streptomycin failed in one of two trials. The parent *Y. pestis* strain had an MIC to gentamicin of 0.5 mg/liter. MIC determinations conducted with the isolates that grew on antibiotic-supplemented agar plates in the nontreatment control arms of both hollow-fiber experiments showed that the bacterial suspensions that were inoculated into the hollow fiber systems did contain a subpopulation of bacteria that had gentamicin MICs of 1 and 2 mg/liter. CLSI guidelines categorize *Y. pestis* isolates with MICs to gentamicin of ≤4 mg/liter as susceptible to this aminoglycoside antibiotic (7). Thus, the strains with decreased susceptibilities to gentamicin were predicted to be killed by the clinically prescribed regimens of gentamicin that were simulated in our experiments.

For the hollow-fiber study in which streptomycin therapy was successful, the colonies that grew on streptomycin-supplemented agars in the nontreatment control arm and in the streptomycin therapy arm prior to initiation of therapy had MICs to this antibiotic of 4 to 8 mg/liter (compared to 2 mg/liter for the wild-type strain). CLSI guidelines suggest that *Y. pestis* isolates with streptomycin MICs of ≤4 mg/liter are susceptible to this antibiotic and isolates with an MIC of 8 mg/liter fall in the "intermediate" category, in that treatment success may occur if high enough drug exposure is achieved in the site of infection (7). In the second trial, streptomycin therapy rapidly killed the wild-type and less-susceptible bacterial populations, suggesting that the clinical regimen of streptomycin of 1 g given every 12 h would be successful against *Y. pestis* infections in humans in which a small proportion of the bacterial population has an MIC to streptomycin as high as 8 mg/liter.

In contrast, in the hollow-fiber experiment in which streptomycin therapy had failed, the streptomycin MICs for isolates in

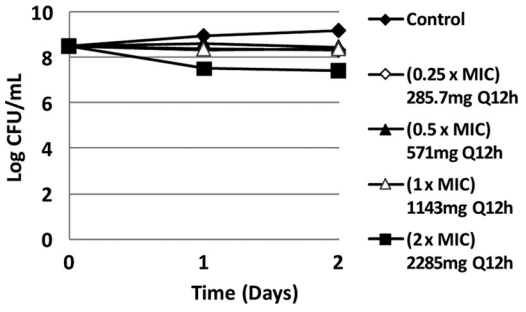

FIG. 3. Time-kill study conducted in flasks showing that the failure of doxycycline to kill *Y. pestis in vitro* is not due to the HFPM but is a general *in vitro* characteristic of the bacterium. The Q12h doses shown in the key for the time-kill study are the dosages of doxycycline needed to generate the 24-h-AUC exposures for the multiples of MICs that were examined in the time-kill experiment.

the nontreatment control arm that grew on agar supplemented with this antibiotic had streptomycin MICs of >256 mg/liter, compared with an MIC of 2 mg/liter for the parent strain. Additional studies using agar plates that contained 128 mg/liter of streptomycin determined that the mutation frequency was −8.46 to −9.03 log CFU for isolates that had MICs to streptomycin of >256 mg/liter. Since the hollow-fiber cartridges were inoculated with 10^9 CFU of bacteria (10 ml of 10^8 CFU of *Y. pestis*), it was a "chance event" whether or not a hollow-fiber system was inoculated with *Y. pestis* isolates with high-level streptomycin resistance.

Genetic studies to define the mechanism of streptomycin resistance that resulted in treatment failure with this antibiotic in one but not the other trial were not conducted in this project. Streptomycin treatment failure was due to *Y. pestis* isolates that had very high MICs to this antibiotic. However, these isolates did not show cross-resistance with gentamicin. Only bacteria that have a mutation in the 30S ribosomal binding site for streptomycin manifest this aminoglycoside-susceptible profile (10, 14, 26). The isolates with MICs for streptomycin of 4 to 8 mg/liter that were detected in the trial in which streptomycin therapy was successful likely expressed aminoglycoside-modifying enzymes with or without efflux pump overexpression (10, 26), since these isolates did have increased MICs to gentamicin.

Gentamicin and ciprofloxacin have been used successfully for the treatment of plague pneumonia in animal models (5) and consistently and rapidly killed *Y. pestis* in the current investigation. Case reports and noncomparative studies suggest that these antibiotics are efficacious for the treatment of plague in humans (18, 23). Our *in vitro* results suggest that these drugs would be equally effective in the treatment of *Y. pestis* infections in humans.

Ampicillin and meropenem have *in vitro* efficacy (5, 12, 27; this study). Little data are available regarding the efficacy of these two antibiotics for the treatment of plague in humans. However, in one murine study, the administration of amoxicillin to mice 24 and 48 h after infection with *Y. pestis* was as efficacious as that of gentamicin (5). Mice with experimental plague pneumonia that were treated with ampicillin beginning 24 h after infection had an 85% survival rate. The regimen given to those mice provided a time-above-MIC of 33% (5), which was much less than the $T>$MIC value of 100% that was simulated in our HFPM. In a separate project, dose fractionation studies conducted in our HFPM showed that a time-above-MIC value of ≤79% of the dosing interval was associated with treatment failure and a time-above-MIC value of ≥92% was predictive of treatment success for ampicillin (21). Since the human clinical regimen of ampicillin of 2 g i.v. given every 6 h that was simulated in the current project generated a time-above-MIC value of 100%, it is likely that the efficacy of this drug in humans would be better than what is predicted from murine infection models.

However, it is important to note that when initiation of ampicillin therapy was delayed to 42 h after infection, the treated mice died more rapidly than the nontreated controls (5). Although not measured, this suggests that the more rapid death was due to endotoxin release by this bacterium as it is killed by beta-lactam antibiotics. Thus, studies need to be conducted to determine if the earlier deaths were indeed due to endotoxin release. Also, studies in other animal species are needed to define whether the severe sepsis induced by beta-lactam therapy for *Y. pestis* in mice occurs in other animal infection models.

In summary, the experiments using the HFPM found that simulated clinical regimens for ciprofloxacin, moxifloxacin, gentamicin, ampicillin, and meropenem were superior to the gold standard, streptomycin, in eradicating a high bacterial burden of *Y. pestis* that could be found in plague septicemia and pneumonic plague. While streptomycin therapy failed due to amplification of resistant mutants in one of two trials, the comparator antibiotics rapidly eradicated the bacteria in both trials. Since streptomycin is currently not available in many countries, including the United States, and since persons with severe plague infections may harbor high bacterial burdens that may include subpopulations with high-level streptomycin resistance, our data suggest that the other antibiotics that were evaluated in the current study should be preferred over streptomycin for the treatment of plague infections.

Importantly, the doses of the drugs examined in this project simulated the mean concentration-time profiles for clinically prescribed regimens. This suggests that at least 50% of the people who would receive the dosages of the antibiotics that were simulated in the HFPM would be cured of plague. Dose-range studies and mathematical modeling (i.e., Monte Carlo simulations) are needed in order to provide a more accurate prediction of the overall efficacy of the evaluated drugs for the treatment of *Y. pestis* infections in humans.

ACKNOWLEDGMENTS

This work was supported by P01AI060908, a grant from NIAID to the Emerging Infections and Pharmacodynamics Laboratory (G. L. Drusano).

The content is solely our responsibility and does not necessarily represent the official views of the National Institute of Allergy and Infectious Diseases, the National Institutes of Health, or the U.S. Army.

We have no conflicts of interest to disclose.

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